AD-A211 156

IFICATION OF THIS PAGE

52

ASSIFIED

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

ASSIFICATION	1b. RESTRICTIVE MARKINGS
ASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT
ATION/DOWNGRADING SCHEDULE	Approved for public release;
ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
	AFOSR-TR- 89-1013

6a. NAME OF PERFORMING ORGANIZATION: SFSU, Tiburon Center for Environmental Studies	6b. OFFICE SYMBOL (If applicable):
6c. ADDRESS (City, State, and ZIP Code): Tiburon, CA 94920, POB 855 3150 Paradise Dr.	7a. NAME OF MONITORING ORGANIZATION: Air Force Office of Scientific Research
	7b. ADDRESS (City, State, and ZIP Code): Bldg 410 Bolling AFB, DC 20332-6448
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: AFOSR	8b. OFFICE SYMBOL (If applicable): NL
8c. ADDRESS (City, State, and ZIP Code): Bldg 410 Bolling AFB DC 20332-6448	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-86-0064
	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO	WORK UNIT ACCESSION NO.
61102F	2312	A5	

DTIC ELECTE AUG 03 1989 S

11. TITLE (Include Security Classification)
Molecular Toxicology of Chromatin

12. PERSONAL AUTHOR(S)
Dr. E. Kun

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM Jan85 TO Jul89	1989, July 4	16

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

During the tenure of this grant period, extending previous studies, a novel physiological regulatory function of a specific DNA binding nuclear protein, ADPRT, has been uncovered, which can explain its cell physiological role as a gene regulator by way of topological modification of DNA structure. The relevance of this approach to the general theme of chromatin toxicology consists in the fact that subtle cellular responses to environmental and genetic factors do not necessarily lead only to a short term lethal outcome, but may result in sustained alteration of gene expression, leading to degenerative diseases and cancer. It was assumed that an understanding of molecular mechanisms that lead to these conditions will allow us to develop molecular pharmacological means to prevent or reverse pathophysiological processes. Results obtained during this research period provide evidence that supports above prediction. As in the past, we proceed simultaneously along molecular enzymological and cell biological lines, and this concerted approach is probably the reason for significant new development.

Briefly, we have developed a rapid isolation procedure for ADPRT (ref. 196) and in possession of relatively large quantities of this protein determined its binding and domain structure (189, 193, 194, 195, 197, 199), which lead to the identification of the most probable predominant cellular function of this protein, consisting of a topological regulation of DNA structure (196). Cell biological correlations (187, 192), notably the inhibition of malignant growth by specific ligands of ADPRT (185, 191) lead to a molecular pharmacological approach to the control of neoplasia and more recently of DNA- and retro-viral DNA synthesis, research that is being conducted beyond the scope of the present report.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL: T. Jan Cerveny	22b. TELEPHONE (Include Area Code): 202-767-5021

DD Form 1473, JUN 86 — Previous editions are obsolete — SECURITY CLASSIFICATION OF THIS PAGE

UNCLASSIFIED

July 4, 1989

Final Progress Report :

"Molecular Toxicology of Chromatin".

AFOSR - 86 - 0064

Period covered : Jan.1985 - July 1989

(including a no-cost extension from Jan.1989 - to date)

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note : 3 years of this research was carried out at UCSF prior to translocation to present quarters.

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Abstract.

AFOSR-TR- 89-1013

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Cell biological correlations (187, 192), notably the inhibition of malignant growth by specific ligands of ADPRT (185, 191) lead to a molecular pharmacological approach to the control of neoplasia and more recently of DNA- and retro-viral DNA synthesis, research that is being conducted beyond the scope of the present report.

List of papers that appeared in print

(no abstracts are listed)

185. Milo,G.E., Kurian,P.,Kirsten,E.,and Kun,E.: Inhibition of Carcinogen Induced Cellular Transformation of Human Fibroblasts by Drugs that Interact with the Poly(ADP-ribose) Polymerase System. FEBS Lett. 179, 332-336, 1985

186. Hakam,A.and Kun,E.: High-Performance Liquid Chromatography of in vitro Synthesized Poly (ADP-Ribose) on Ion-Exchange Columns, Separation of Oligomers of Various Chain Lengths and Estimation of Apparent Branching. J.Chromatogr. 330, 287-298, 1985.

187. Kirsten,E.,Jackowski,G.,McLick,J.,Hakam,A.,Decker,K.,and Kun,E.: Cellular Regulation of Poly ADP-ribosylation of Proteins. 1. Comparison of Hepatocytes, Cultured Cells and Liver Nuclei, and the Influence of Varying Concentrations of NAD. Exper.Cell Res. 161, 41-52, 1985.

188. Kun,E., Minaga,T., Kirsten,E., Hakam,A., Jackowski,G., Tseng,A.,jr.and Brooks,M.: "Possible Participation of Nuclear Poly(ADP-Ribosyl)ation in Hormonal Mechanisms" in: Biochemical Actions of Hormones, Vol. XIII (ed.: Litwack, G.) Chapter 2. Acad.Press, New York, 1986.

189. Bauer,P.I.,Hakam,A.and Kun,E.: Mechanisms of Poly (ADP-ribose) Polymerase Catalysis; Mono-ADP-Ribosylation of Poly (ADP-Ribose) Polymerase at Nanomolar Concentrations of NAD. FEBS Lett. 195, 331-338, 1986.

190. Hakam,A.,McLick,J.and Kun,E.: Simultaneous Determination of Mono- and Poly(ADP-Ribose) in vivo by Tritium Labeling and Direct High Performance Liquid Chromatography Separation. J.Chromatogr. 395, 275-284, 1986.

191. Tseng,A.,jr., Lee,W.F.M., Kirsten,E., Hakam,A., McLick,J., Buki,K.G.,and Kun,E.: Prevention of Tumorigenesis of Oncogen-Transformed Rat Fibroblasts with DNA-Site Inhibitors of Poly(ADP-Ribose) Polymerase. Proc.Nat.Acad.Sci.(USA) 84, 1107-1111, 1987

192. Sooki-Toth,A.,Asghari,F.,Kirsten,E.and Kun,E.: Cellular Regulation of Poly ADP-Ribosylation of Proteins.II. Augmentation of Poly ADP-Ribose Polymerase in SV40-3T3 Cells Following Methotrexate Induced G1/S Inhibition of Cell Cycle Progression. Exp.Cell Res. 170, 93-102, 1987.

193. McLick,J.,Bauer,P.I.,Hakam,A.,and Kun,E.: Covalent Binding ofCarbamoylbenzenediazonium Chloride to Deoxyguanine Bases of DNA Resulting in Apparent Irreversible Inhibition of Poly ADP-Ribose Polymerase at the Nicotinamide Site. Biochemistry 26, 2226-2231, 1987.

194. McLick,J.,Hakam,A.,Bauer,P.I.,Kun,E.,Zacharias,D.and Glusker,J.: Benzamide-DNA Interactions: Deductions from Binding, Enzyme Kinetics and from X-Ray Structural Analysis of a 9-Ethyladenine-Benzamide Adduct. Biochim.Biophys.Acta 909, 71-83, 1987.

195. Hakam,A.,McLick,J.,Buki,K.G.,and Kun,E.: Catalytic Activities of Synthetic Octadeoxyribonucleotides as Coenzymes of Poly (ADP-Ribose) Polymerase and the Identification of a New Enzyme Inhibitory Site. FEBS Lett. 212, 73-78, 1987.

196. Buki,K.G.,Kirsten,E.,and Kun,E.: Isolation of Adenosine Diphosphoribosyl Transferase by Precipitation with Reactive Red 120 Combined with Affinity Chromatography. Analyt.Biochem.167, 160-166, 1987.

197. Kun,E.: "Probable Macromolecular Mechanism of Gene Regulation by Adenosine Diphoshoribosyl Transferase (ADPRT)" in: Proceedings of the Ist Internat.Symposium on Post-Translational Modification of Proteins and Ageing. pp. 613-626 (Ed.:V.Zappia, Univ.Naples) Plenum Press, 1988.

198. Sastry,S.S.and Kun,E.: Interaction Between DNA, Poly(ADP-Ribose) Polymerase and Histones. J.Biol.Chem. 263, 1505-1512, 1988.

199. Buki,K.G.and Kun,E. : Polypeptide Domains of ADP-Ribosyltransferase Obtained by Digestion with Plasmin. Biochemistry 27, 5990-5995, 1988.

200. Sastry,S.S., Buki,K.G.and Kun,E. : The Binding of Adenosine Diphosphoribosyl Transferase to the Termini and Internal Regions of Linear DNAs. Biochemistry, in press.

Detailed Report.

The rationale followed here is to analyze significant aspects of published work, IN ORDER TO delineate progress and forecast future plans.

I. Molecular Structure and Catalytic Activities of ADPRT.

One of the main obstacles to progress in the field of the molecular enzymology of ADPRT was the clumsiness of its isolation in molecularly homogeneous form. The reasons for this difficulty were poor extraction procedures and slow purification steps which resulted in poor yields of an apparently unstable protein. Apparent instability is most likely due to contaminating proteases, which appear to be specific for ADPRT, thus a rapid decay of the enzyme molecule ensues during slow isolation procedures reported by others in the past. Drs. Buki and Kirsten developed a two prong affinity column system, which is taking advantage of both DNA specific and NAD specific sites on the enzyme protein. The Reactive Red 120 and Benzamide affinity columns, when attached in tandem, can rapidly and efficiently produce more than 95% homogenous ADPRT protein within a short working period (2-3 days) with high yields (ref.196), approximating the high intracellular concentrations of this protein, determined by specific antibody binding (ELISA) tests in intact cells. The development of this technology was the key to further molecular and catalytic studies.

Binding Studies of ADPRT.

Crucial to the understanding of biological activity is the precise mechanism of ligand binding to ADPRT. This work, which is also the theme of ongoing and future work, commenced during this grant period and consisted of an analysis of the binding mechanism of benzamide to ADPRT and to its coenzymic DNA (194). Benzamide was known as a competitive inhibitor of ADPRT, competing for the acid amide portion of of the nicotinamide component of NAD. We have described in 1983 (PNAS 80,7219-7223, 1983) an antineoplastic effect of benzamide in human fibroblasts, that was hard to explain on the basis of "metabolic" enzymatic inhibitory effect on ADPRT alone. In collaboration with the X-ray crystallographers Drs. Zacharias and Glusker (Philadelphia), McLick et al. investigated two binding aspects, namely a direct H-bonded interaction with DNA, as modeled by a co-crystal of benzamide and ethyladenine, and the binding of benzamide to ADPRT protein at its DNA site, which is distinctly different from its NAD site. This extensive paper (194) demonstrates that the cell biological action of benzamide (anticancer effect) need not to be related to the "metabolic" activity of ADPRT, but to a DNA-binding related activity, which we recently identified as the DNA-topological activity (198), thus binding studies predicted a new function that is now becoming intelligeable in molecular terms.

A further development, originating in this binding and X-ray diffraction work, was then elaborated by McLick, Bauer, and Hakam

(193). It was shown that binding at the nicotinamide site of ADPRT coincided with a direct association of ADPRT inhibitors with deoxyguanidine sites of coenzymic DNA as shown by covalent affinity labeling, enzyme kinetics and NMR and HPLC techniques. The distance between the NAD and DNA binding sites on the protein was 8.5 Å, a molecular dimension which has predictive significance in constructing ADPRT ligands with potential pharmacological (anticancer) action, since molecular modeling will allow us the construction (synthesis) of drugs that "fit" into the sites identfied above. These results had two major aereas of impact. First, it became increasingly certain that a DNA binding site and an NAD binding site on ADPRT may have different physiological significance. Second, from bio-organic chemical priciples predictions can be made for the synthesis of highly effective, but presumably low-toxicity molecules, if the polypeptide structure of ADPRT and of binding DNA structures are better understood. This idea guides present efforts.

Critical to this research was the understanding of the coenzymatic role of DNA to ADPRT. From existing studies of others, the general dogma professed that broken DNA serves as a cofactor to ADPRT. If this is true then relatively short linear DNA fragments should exert the same catalytic function on ADPRT regardless of their base composition. As shown by Hakam, McLick and Buki (195) this was not the case, since the catalytic efficiency of synthetic octameric duplex oligodeoxyribonucleotides was influenced by their base composition, thus a structural internal element of DNA was discovered that preceeded our direct demonstration of terminal and internal binding of

ADPRT (current research). The advantage of the specific coenzymic function of synthetic octamer duplexes, instead of undefined "coenzymic DNA" that co-isolates with ADPRT (196) is becoming of great importance in ongoing work related to molecular mechanisms.

The molecular anatomy of ADPRT was further clarified by Buki and Kun (199) by dissecting this molecule into peptidic fragments with the aid of the protease plasmin. Plasmic fragments defined a 29 kDa terminal basic polypeptide (containing a blocked amino terminus), a 36 kDa (duplex) basic peptide duett, differing only in one lysine residue, that specifically binds DNA, and a 56 kDa neutral polypeptide that was cut by plasmin further into 14 and 42 kDa fragments. The NAD binding site and catalytic center(s) are associated with the 56 and 42 kDa peptides, and the 14 kDa, which is lost during protease action, has now been synthesized chemically (collaboration with Cetus) and its precise role is being now determined (ongoing research). The polypeptide structural studies further yielded significant results regarding polypeptide sequences, from which we synthesized various DNA probes for the identification of the cDNA for ADPRT in E.coli containing inserted cDNA fragments. We succeeded to obtain a cDNA probe for ADPRT, however further work was curtailed by receiving no continued grant support from AFOSR to complete the cloning project.

However, significant progress was initiated from the isolation of the plasmic fragments : antibodies were developed against each peptide and their effect on DNA, NAD and histone binding to ADPRT

determined, a subject which is part of our present research.

Perhaps the most significant result of this work was the discovery that NAD binding alone induces large conformational changes in ADPRT, which prohibit DNA binding. This macromolecular mechanism (NAD induced conformational change in ADPRT) is of utmost significance in explaining the action of other ADPRT ligands on cell function (see below).

Catalytic Mechanisms of ADP-Ribosylation.

Poly ADP-ribosylation enzymology has been the subject of extensive studies in many laboratories, and an ADPR-glutamate-carboxyl ester binding group has been identified, presumably representing the structure of protein-ADPR adducts. This ester bond is hydrolyzed at pH 9.5 - 12, or by base-hydroxylamine, - thus behaves like a fairly stable ester. Investigating mechanisms of cellular ADP-ribosylations in quasi-physiological systems (cell culture) we found (187) that at nanomolar concentrations a highly base unstable mono-ADP-ribose-ADPRT adduct was formed as an initiator ADPR-Enzyme adduct. The nature of this new molecular species was further studied by Bauer and Hakam (189). It was ascertained that the bond involved in the formation of mono-ADPR-enzyme adduct is unstable above pH 6.0 and OH^- ions rapidly hydrolyze it into ADPR + free enzyme, accounting for the NAD-glycohydrolase activity of ADPRT, a "side-reaction" catalyzed by this enzyme which has been known but remained unexplained. The importance of the mono-ADPR-enzyme adduct consists of the fact that it explains trans-ADP-ribosylations (to either stable carboxyl esters on the ADPRT molecule or to

histones). We also utilized this mono-ADPR reaction in quantitation of ADPRT (192), results which we now confirmed by direct immunotransblot assays. Furthermore, on the basis of this work (187, 189) we presently identified the chemical structure of the ADPR-enzyme bond to be an energy rich imidazolyl-ADPR, a work presently pursued.

As a technological advance Hakam et al. (185) further developed HPLC methodology that was based on previously reported studies from our laboratory. The HPLC method is especially useful for enzyme mechanism studies and also foe in vivo polymer etc. analyses.

The Second Molecular Activity of ADPRT.

It was becoming increasingly apparent that the metabolic enzymological activity of ADPRT (which is poly ADPR synthesis from NAD) by itself has a limited capability in explaining a physiological role of this protein in normal cell function. In contrast stands the DNA-damage related cell-suicide mechanism, which maximally and non-specifically activates ADPRT and is operative only under catastrophic artefactual toxic conditions. The complex DNA, histone and NAD binding capability of the ADPRT molecule also predicted that ADPRT may function as a DNA binding protein intracellularly.

The work of Sastry and Kun (198) provided direct evidence that identified a new molecular activity of ADPRT. First it was shown that binding of ADPRT to restricted genomic DNA is greatly

augmented (following a cooperative type kinetics) by histones. This cooperative binding mechanism followed an ordered addition mechanism : either ADPRT or histones have to bind to DNA before the second macromolecule (histone or ADPRT, respectively) is added. This macroscopic order of addition kinetics has interesting implications in the nucleosomal regulation of gene expression, as we now observe in recent work. The binding of ADPRT to DNA has a remarkable effect on DNA conformation, an observation which defines a novel molecular activity of ADPRT. When closed circular SV40 DNA, which does not serve as a metabolic coenzyme to ADPRT (DNA termini are required for this coenzymatic function) binds ADPRT, the circular DNA condenses, as assayed in a topo-I coupled gel system (cf. 198). It is apparent from the gel pattern that enzyme-DNA intermediates are formed, identifying a gradual process of topological transition from relaxed to the condensed state. NAD binding inhibits the topological effect of ADPRT, consistent with our observation that NAD has a large conformational effect on ADPRT protein structure, and it seems plausible that the ADPRT-NAD complex induced ADPRT conformation is structurally prohibited to alter DNA topology. However, ligands of ADPRT which bind not only at the NAD site but at additional enzyme sites (cf. 193, 194, 195) re-convert ADPRT to DNA topological activation. This model explains the cell biological action of ADPRT ligands, since the in vivo inactive DNA topological capability of ADPRT (inhibited by NAD binding) is inactivated by ADPRT ligands (other than NAD) and the ensuing conformational change in DNA structure then produces either gene-

repressions or activations, depending on the specific state of DNA in a particular cell type (cf.197). A confirmation of this model also emerged from further ADPRT-DNA binding analyses, carried out with the nuclease-protection technique (cf.200). It was shown in linearized ds-DNAs that ADPRT binds non-selectively to DNA termini (producing polymerase activity of ADPRT) and highly selectively to internal DNA domains, that cannot be characterized by specific DNA sequences, but rather by unusual (replication oriented ?) structures. In all cases ADPRT binding profoundly changes DNA structures, as assayed in the nuclease protection tests, thus the ligand ADPRT is not a passive binder, but produces significant structural changes, undoubtedly of basic cell physiological importance.

Our present work is aimed at the detailed mechanisms underlying these macromolecular interactions.

Cell biological studies comprise experiments that are the consequence of our original discovery in 1983, demonstrating antineoplastic activity of the ADPRT ligand benzamide. We subsequently reported in 1985 (ref.185) that pretreatment of human fibroblasts with ADPRT ligands (benzamide or 1,2-benzopyrone etc.) rendered these cells resistant to carcinogenesis by chemical carcinogens. This phenomenon has no explanation, unless the DNA topological catalysis of ADPRT is invoked, as discussed above. To ascertain the antineoplastic mechanism of ADPRT ligands we constructed a rat cell to contain a dexamethasone -inducible gene structure (pMTV-EJ-ras plasmid) and determined the effect of the ADPRT ligands:benzamide and 1,2-

benzopyrone on carcinogenic growth both in vivo and in vitro (191). Inhibition of tumorigenic growth was obtained without cell killing, indicating the conversion by ADPRT ligands of malignant growth to non-malignant proliferation. Surprisingly, oncogene expression was not altered at the time growth was stopped, demonstrating that a basic regulatory mechanism of DNA replication, conversion of malignant to non-malignant mode, is being primarily regulated by the drug induced topological effects of ADPRT.

The molecular details of these clearly basic discoveries are being studied during our current support from AFOSR.